

### REMARKS

Upon entry of the present amendment, claims 1-4, 11-13, and 39-43 will be pending. Applicants have amended claims 1 and 3, and added new claims 39-43. Claims 9, 10, and 14-38 have been cancelled. Support for the amendments and new claims can be found throughout the application as filed, e.g., at page 28, line 29, to page 29, line 11; page 30, line 22, to page 32, line 13; the Examples and Figures; and the claims as filed, *inter alia*. No new matter has been added.

#### Objections to the Specification

The disclosure was objected to at page 2 of the Office Action mailed January 2, 2009 (the "Office Action"), because the specification refers to various colors in the drawings, but the drawings were not filed in color. Applicants have amended the specification as shown above to delete the references to colors, or to indicate that the color was present in the original, as appropriate. Applicants submit that these amendments obviate the objection to the specification and request withdrawal thereof.

#### Sequence Compliance

Also at page 2 of the Office, the Examiner alleged that the application failed to comply with the requirements of 37 CFR §§ 1.821-1.825, for failing to submit a sequence listing including the sequences present in FIGs. 1, 3, 7-36 and 39. Applicants respectfully direct the Examiner's attention to the Applicants' response to the Notice of Missing Requirements, which was filed February 15, 2007, and included a Sequence Listing in compliance with 37 CFR §§ 1.821-1.825 and a Preliminary Amendment directing entry thereof. Applicants note that the Sequence Listing and Amendment are present in PAIR. If such Amendment was not entered, Applicants request that it be entered at this time. Furthermore, if that sequence listing was in some way defective, Applicants request that the specific defects be identified so that Applicants can correct them.

Applicants do note that the sequence shown in Figure 1 was not included in the sequence listing, as it falls outside the sequence listing rules (nucleic acid sequences with fewer than ten

nucleotides), see 37 CFR § 1.821 ("Nucleotide ... sequences as used in §§ 1.821 through 1.825 are interpreted to mean ... unbranched sequence of ten or more nucleotides").

Therefore, Applicants submit that the application does comply with the requirements of 37 CFR §§ 1.821-1.825, and request withdrawal of the objection thereunder.

Claim Rejections - 35 USC § 112 - 1<sup>st</sup> paragraph

Pending claims 1-4 and 11-13 were rejected as allegedly lacking enablement. The Examiner noted that the specification was enabling for:

1) a method for determining whether a test compound is a candidate SKN-1-mediated oxidative stress response-activating compound, comprising: (a) providing a first nematode capable of expressing a SKN-1 polypeptide and containing at least one transgene comprising an oxidative stress resistance gene promoter operably linked to a reporter gene *wherein the promoter comprises a SKN-I binding site effective at directing constitutive and stress-induced expression*; (b) contacting the first nematode with the test compound; and (c) determining whether expression of said at least one transgene is increased in said first nematode *compared to a control nematode not contacted with said test compound*, wherein an increase in expression of the transgene in the nematode contacted with the test compound compared to the control nematode indicates that the test compound is a candidate SKN-1-mediated oxidative stress response activating compound (claim 1), and

2) a method for determining whether a test compound is a candidate SKN-1-mediated oxidative stress response-inhibiting compound, comprising: (a) providing a first nematode capable of expressing a SKN-1 polypeptide and containing at least one transgene comprising an oxidative stress resistance gene promoter operably linked to a reporter gene *wherein the promoter comprises a SKN-I binding site effective at directing constitutive and stress-induced expression*; (b) contacting the first nematode with the test compound; (c) subjecting the nematode to conditions that are known activate the SKN-1 mediated oxidative stress response in the absence of the test compound, and (d) determining whether expression of said at least one transgene is decreased in said first treated nematode *compared to a control nematode not contacted with said test compound*, wherein a decrease in expression of the transgene in the nematode contacted with the test compound compared to the control nematode indicates that the test compound is a candidate SKN-1-mediated oxidative stress response-inhibiting compound (claim 3), and

3) said methods further comprising determining if the test compound binds either GSK-3 or SKN-1 or is a potentially inhibitor of GSK-3 or SKN-1 activity (claims 2 and 4). (*Emphasis in original*)

However, the Examiner further indicated that the specification

does not reasonably provide enablement for the claimed method wherein the oxidative stress promoter does not comprise SKN-1 responsive elements or wherein there is no control for comparison or for definitively determining that the test compound is an inhibitor of GSK-3 or SKN-1 activity.

Applicants have amended claims 1 and 3 to specify that the oxidative stress resistance gene promoter comprises a SKN-1 binding site and is effective at directing SKN-1-induced gene expression. In addition, Applicants have amended claims 1 and 3 to specify comparing the treated nematode to a control nematode not treated with the test compound. Applicants therefore submit that claims 1 and 3 as amended satisfy the enablement requirement.

Regarding the rejection of claims 2 and 4 as allegedly lacking enablement, Applicants explicitly do not concede that the claims as written were not enabled, and note that a number of assays were known in the art at the time of filing for determining whether a compound actually inhibited GSK-3 or SKN-1. However, in the interest of expediting prosecution, Applicants have amended claims 2 and 4 to recite methods that include determining whether the compound enhances or inhibits SKN-1-mediated expression of an oxidative stress resistance gene in a cell. Suitable methods are well known in the art and are also described in the specification, see, e.g., the Examples. Applicants further submit that new claims 44 and 45, which recite methods that comprise determining whether the compound increases (44) or decreases (45) Nrf-1-mediated expression of an oxidative stress resistance gene in a mammalian cell, as compared to a control cell, are also amply enabled; the identity of genes in the stress resistance pathway in mammals is also well known (see, e.g., page 30, line 19, to page 32, line 9; page 34, lines 10-23 and references cited therein; and page 51, line 26, to page 52, line 7 and references cited therein, especially Hayes and McMahon, *Cancer Lett* 174: 103-113 (2001), *copy submitted herewith*), as are methods of determining whether a compound affects gene expression levels.

For at least these reasons, Applicants submit that the pending claims are amply enabled, and request withdrawal of the rejections under 35 U.S.C. § 112, first paragraph.

Claim Rejections - 35 USC § 112-2<sup>nd</sup> paragraph

Pending claims 1-4 and 11-13 were rejected at pages 7-8 of the Office Action for allegedly being indefinite in the recitation of "the transgene." Applicants have amended independent claims 1 and 3 to recite "the one or more reporter transgenes" and "the reporter transgene" to clarify that the same transgene is being referred to, i.e., the transgene comprising an oxidative stress resistance gene promoter, and a reporter gene operably linked thereto.

In light of these amendments, Applicants submit that the pending claims are clear and definite and request withdrawal of the rejection under 35 U.S.C. § 112, second paragraph.

Conclusion

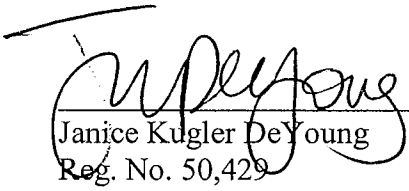
In light of the amendments and arguments made herein, Applicants submit that the pending claims are allowable, and request early and favorable action thereon. If the Examiner feels that it would expedite allowance of this application, she is invited to telephone the undersigned at (617) 956-5985, Monday, Wednesday, or Friday.

No fees are believed to be due. Please apply any charges or credits to deposit account 06-1050.

Respectfully submitted,

Date:

February 28, 2007

  
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Mini review

# Molecular basis for the contribution of the antioxidant responsive element to cancer chemoprevention

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## Abstract

This article provides an overview of the mechanisms by which cancer chemopreventive blocking agents increase the expression of detoxication and antioxidant genes. These agents all appear capable of transcriptionally activating a gene battery that includes NAD(P)H:quinone oxidoreductase, aldo-keto reductases, glutathione *S*-transferases,  $\gamma$ -glutamylcysteine synthetase, glutathione synthetase and heme oxygenase. Gene induction occurs through the antioxidant responsive element (ARE), a process that is dependent on the Nuclear Factor-Erythroid 2p45-related factors, Nrf1 and Nrf2. Under basal conditions, these basic region leucine zipper (bZIP) transcription factors are located in the cytoplasm of the cell bound to Keap1, and upon challenge with inducing agents, they are released from Keap1 and translocate to the nucleus. Within the nucleus, Nrf1 and Nrf2 are recruited to the ARE as heterodimers with either small Maf proteins, FosB, c-Jun, JunD, activating transcription factor 2 (ATF2) or ATF4. The role of protein kinases in transducing chemical stress signals to the bZIP factors that affect gene induction through the ARE is discussed. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

**Keywords:** Chemoprevention; Detoxication; Antioxidant responsive element; Nrf1; Nrf2; Cytochrome P450; Glutathione *S*-transferase; Mitogen activated protein kinase; Carcinogenesis; Protein kinase C

## 1. Introduction

A wide range of chemicals, both naturally-occurring and synthetic, can protect against the development of cancer. These include coumarins, diterpenes, dithiolethiones, indoles, isothiocyanates, lactones, organosulphides and phenols (for a review, see [1]). Compounds with cancer chemopreventive properties have been subdivided into blocking agents and suppressing agents on the basis of the stage during carcinogenesis at which they act [2]. Blocking agents

prevent carcinogens from modifying DNA and causing mutations. This is usually achieved by increasing the expression of detoxication and antioxidant enzymes in target tissues, though alterations in the pharmacokinetics of xenobiotics may also serve to protect against tumourigenesis. Such responses are thought to represent a form of cellular adaptation to chemical and oxidative stress [1]. By contrast, suppressing agents inhibit the later promotion and progression stages of neoplastic disease. Their actions include antagonism of oncogenes, activation of tumour suppressor proteins, inhibition of angiogenesis, stimulation of apoptosis or terminal differentiation, and modulation of arachidonic acid cascades.

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## 2. Induction of detoxication and antioxidant genes as mechanisms of cancer chemoprevention

It was first shown over 20 years ago that chemopreventive blocking agents increase the hepatic levels of enzymes involved in drug metabolism [2,3]. Cytochrome P450 (CYP), NAD(P)H:quinone oxidoreductase (NQO), aldehyde dehydrogenase, aldo-keto reductase, glutathione *S*-transferase (GST), uridine diphosphate (UDP)-glucuronosyl transferase (UGT), and microsomal epoxide hydrolase represent some of the enzymes that are now known to be induced by chemopreventive agents [3–6]. These findings suggest that an enhanced detoxification capacity represents a mechanism by which compounds with anticancer properties confer at least some of their beneficial effects.

Besides influencing drug metabolism, it is becoming recognized that blocking agents also induce antioxidant proteins and enzymes involved in the inflammatory response. Such proteins include the catalytic heavy subunit and the regulatory light subunit of  $\gamma$ -glutamylcysteine synthetase (GCS<sub>h</sub> and GCS<sub>l</sub>), glutathione synthetase,  $\gamma$ -glutamyl transpeptidase, heme oxygenase (HO), the heavy and light subunits of ferritin, peroxiredoxin MSP23, and leukotriene B<sub>4</sub> 12-hydroxydehydrogenase [4,6,7]. Thus, increases in antioxidant status, scavenging of free radicals, and detoxification of both reactive oxygen species and the cytotoxic metabolites they generate through damaging and degrading macromolecules also represent mechanisms by which anticancer agents may protect against neoplasia [8].

Recognition that certain blocking agents serve to increase NQO and GST activities but not arylhydrocarbon hydroxylase activity (CYP1A1 and CYP1A2), whereas others induce all three activities, led Prochaska and Talalay [9] to refer to the former compounds as monofunctional inducers and the latter compounds as bifunctional inducers. Subsequent characterization of the 5'-flanking regions of the rat *NQO1*, *GSTA2* and *CYP1A1* genes has revealed that monofunctional inducers transcriptionally activate the expression of these genes through the antioxidant responsive element (ARE), whilst bifunctional inducers act through the ARE as well as the xenobiotic responsive element (XRE) [1]. The fact that both monofunctional and bifunctional inducers stimulate

the ARE suggests that this enhancer plays a central role in the anticancer actions of such agents.

Biotransformation of blocking agents is often essential for them to affect gene induction. Evidence indicates that activation of ARE-driven gene expression requires a metabolic stimulus. Blocking agents that work through the ARE either possess a thiol-active moiety or are converted within the body to a metabolite bearing such a group [1,10]. For example,  $\beta$ -naphthoflavone ( $\beta$ -NF) requires to be oxidized by CYP1A1 to a putative quinone- or hydroquinone-containing metabolite [11]. Similarly, butylated hydroxyanisole (BHA) requires to be converted, through *O*-demethylation catalyzed by another CYP isoenzyme, to *tert*-butylhydroquinone (t-BHQ), and ethoxyquin is oxidized to the  $\alpha,\beta$ -unsaturated carbonyl-containing compound 2,2,4-trimethyl-6-quinolone [3,12]. In addition, a significant number of blocking agents exist, including isothiocyanates, that stimulate the ARE directly [13]. Fig. 1 shows the *cis*-acting elements and metabolic events by which anticarcinogens regulate gene expression. It should be noted that as indoles only appear to stimulate XRE-driven gene expression, and not ARE-driven gene expression (at least in LS-174 and Caco-2 human colon cells), they ought not to be classed as bifunctional inducers [14].

## 3. Identification of the ARE

The ARE was first characterized by Pickett and his colleagues as an enhancer within a 41 bp region from the 5'-flanking region of the rat *GSTA2* gene that was responsive to  $\beta$ -NF [15]. Initially, the enhancer was referred to as a  $\beta$ -NF responsive element [11,15], but was re-designated as an ARE once it was recognized to respond not only to  $\beta$ -NF but also to phenolic antioxidants that undergo redox cycling [16]. Furthermore, the ARE responds to H<sub>2</sub>O<sub>2</sub> and hypoxia, as well as to  $\alpha,\beta$ -unsaturated carbonyls and hydroperoxides that are typically generated during oxidative stress [16–19]. This enhancer is therefore now thought to play an important role in the regulation of antioxidant genes in response to exposure to pro-oxidants [1,8].

Deletion and mutational analyses of the rat *GSTA2* promoter identified the core sequence required for basal and/or inducible activity of the ARE as 5'-

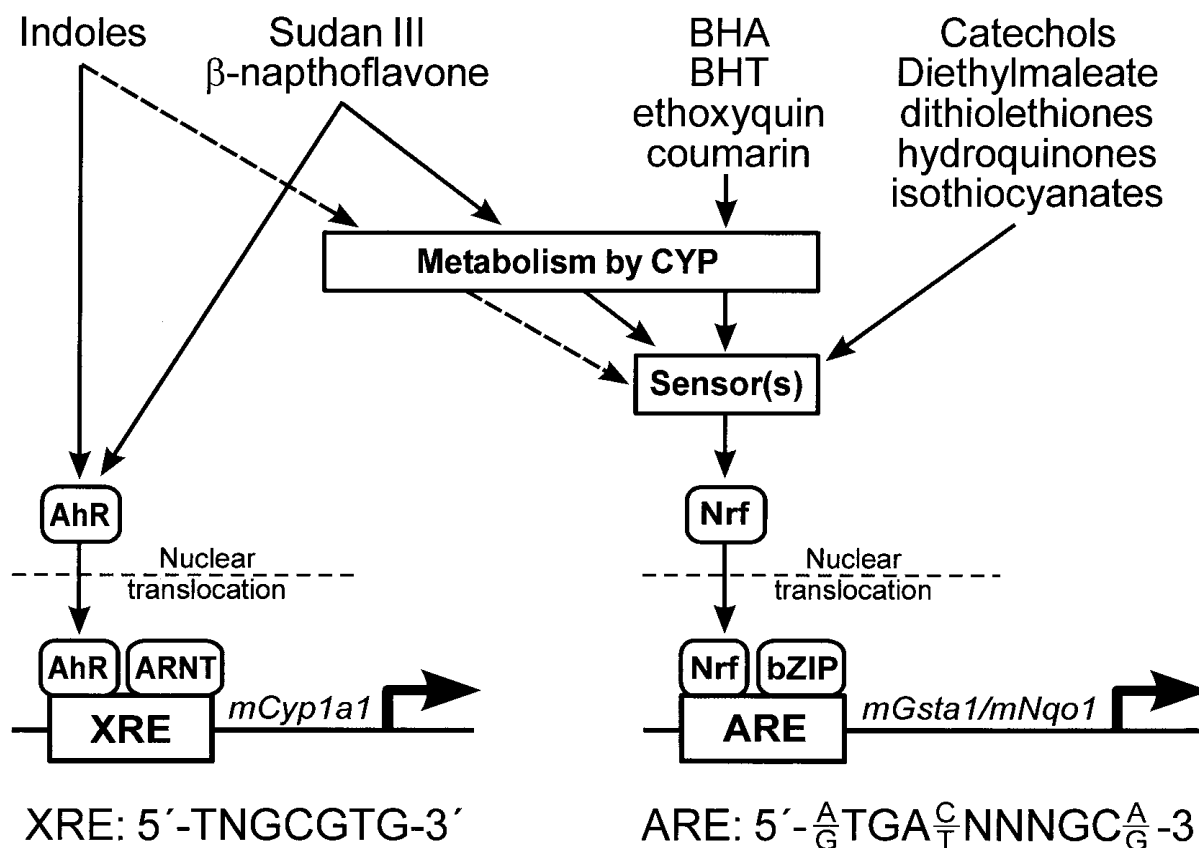
**Bifunctional inducers****Monofunctional inducers**  
(pro-drugs) (direct-acting drugs)

Fig. 1. Regulation of XRE- and ARE-driven gene expression by chemopreventive blocking agents. This diagram illustrates the various metabolic pathways, transcription factors and *cis*-acting elements that are involved in transcriptional activation of genes by monofunctional and bifunctional inducers. Some of the chemicals that act directly as monofunctional inducers are listed, as are others that require biotransformation before they can stimulate the ARE (see right-hand side of the figure). In addition, the ARE can be stimulated by certain Ah receptor ligands, such as Sudan III and β-NF, once they have been oxidized by CYP isoenzymes; these are referred to as bifunctional inducers [9]. Indoles are considered to be chemopreventive agents, and although they cannot activate the ARE directly [14], it is unclear whether their metabolites are capable of stimulating this enhancer. Thus, the hatched arrow from indoles on the left-hand side of the figure to the ARE only represents a tentative assignment. The consensus sequence for the ARE, shown at the bottom, is described elsewhere [15–17]. The consensus sequence for the XRE is taken from Ref. [18]. Since AREs recruit either Nrf1 or Nrf2 as heterodimers with small Maf proteins, FosB, c-Jun, JunD, ATF2 or ATF4, the complex above ARE in the diagram is simply Nrf-bZIP (see below for further details).

TGACNNNGC-3' [16]. Subsequent functional analysis has shown that the T nucleotide in the 5' part of the ARE is essential for both its basal and inducible activity, whereas the adjacent G is only essential for induction [20]. Mutation of the other more proximal nucleotides attenuates, but does not abolish, ARE activity [20].

Within the promoter of the murine *Gsta1* gene, a 41 bp region exists that shares 95% sequence identity

with the ARE-containing region in rat *GSTA2* [1,21]. The enhancer flanking *mGsta1* was originally called an electrophile responsive element (EpRE) because it can be stimulated by dimethyl fumarate and *trans*-4-phenyl-3-buten-2-one, as well as by t-BHQ and β-NF [21].

ARE enhancers have been found in the promoters of the rat and human *NQO1* genes [22,23], and in the genes encoding the human GCS<sub>n</sub> and GCS<sub>i</sub> subunits

[24,25]. Also, the ARE has been demonstrated to be present in the 5'-flanking region of the mouse and human HO-1 genes [26,27], where it has been called a stress responsive element (StRE) because it responds to heme, heavy metals, arsenite and 12-*O*-tetradecanoylphorbol-13-acetate. This enhancer has similarly been identified in the promoters of the murine metallothionein 1 gene [28], the rat inducible nitric oxide synthase gene [29] and the rat  $\gamma$ -glutamyl transpeptidase gene [30]. The human spermidine/spermine *N*<sup>1</sup>-acetyltransferase gene may also contain an ARE, but in this instance, it has been called a polyamine-responsive element [31].

Comparison between the ARE enhancers in the promoters of *rGSTA2*, *mGsta1*, *rNQO1*, *hNQO1* and *rGSTP1* allowed a consensus sequence to be proposed, namely, 5'-T<sup>A</sup>/CANN<sup>A</sup>/GTCG<sup>C</sup>/TNNNGC<sup>A</sup>/G-3' [17]. Based on this consensus sequence, Wasserman and Fahl [17] employed a bioinformatics approach to identify ARE-like motifs in the promoters of rodent genes encoding the GSH transporter, ferritin-L, tyrosinase and interleukin 6. The significance of these putative enhancers has yet to be established.

#### 4. Role of basic region leucine zipper transcription factors in regulation of detoxication/antioxidant genes and sensitivity to carcinogenesis

Similarity exists between the ARE and the DNA-binding motifs of transcription factors within the basic region leucine zipper (bZIP) superfamily. Friling et al. [21] highlighted the fact that part of the core ARE bears some resemblance to the 5'-TGA<sup>C</sup>/GT<sup>A</sup>/CA-3' DNA recognition motif for AP-1. Although the ARE consensus sequence is clearly distinct from the classic AP-1 binding site, it is nevertheless related to the DNA-binding sites of other bZIP transcription factors, such as Nuclear Factor-Erythroid 2 (NF-E2), 5'-A<sup>A</sup>/GTGA<sup>C</sup>/GTCA<sup>A</sup>/G-3' [32], and the viral oncoprotein v-Maf, 5'-TGCTGACTCAGCA-3' [33].

NF-E2p45 is the founding member of a family of cap 'n' collar (CNC) bZIP transcription factors that includes NF-E2-related factors 1, 2 and 3 (Nrf1, Nrf2 and Nrf3) [32,34–36] and the more distantly related Bach1 and Bach2 [37]; Fig. 2 shows a cartoon depicting the conserved Nrf2-erythroid cell-derived protein with CNC homology (ECH) homology

domain 2 (Neh2), CNC and bZIP domains of p45 NF-E2, Nrf1, Nrf2 and Nrf3; the Neh2 has been defined by Itoh et al. [38]. The possibility that any of the CNC bZIP factors might mediate gene regulation through the ARE was first tested by Venugopal and Jaiswal [39]. These investigators demonstrated, by transient co-transfection of expression vectors for various bZIP proteins and reporter gene constructs into HepG2 cells, that overexpression of both Nrf1 and Nrf2 significantly increases ARE-driven gene expression. Furthermore, electrophoretic mobility

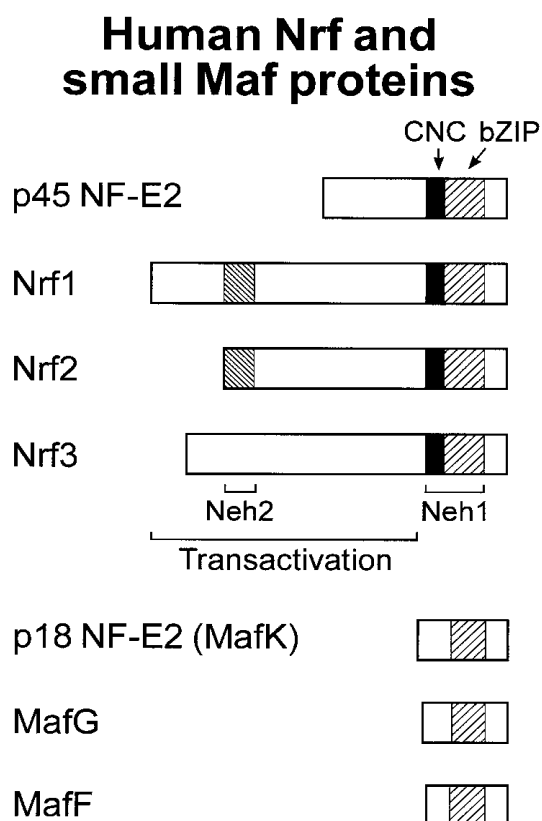


Fig. 2. Location of structural domains in the p45 subunit of NF-E2 and related factors. Molecular cloning of p45 NF-E2, Nrf1, Nrf2 and Nrf3 revealed that the C-terminal part of each protein contains a cap 'n' collar (CNC) domain (shown in black) immediately adjacent to a bZIP domain (shown in diagonal lines, from top right to bottom left). Together, the CNC and bZIP domains have been referred to as Neh1 by Yamamoto and his colleagues [38]. The Neh2 domain can be identified as conserved region of about 90 amino acids within the N-terminal third of Nrf1 and the N-terminus of Nrf2 that is responsible for the negative regulation of at least Nrf2 by Keap1 (represented by boxed area containing diagonal lines, from top left to bottom right) [38]. For comparison, the small Maf proteins are shown as bZIP factors that lack both the CNC and Neh2 domains.



super-shift assays demonstrated that hNrf1 was capable of binding the ARE found flanking *hNQO1* [39], and that rNrf2 can bind the ARE in the promoters of *rGSTA2* and *rNQO1* [20].

The physiological role of Nrf1 has been the subject of several investigations. Nrf1 clearly performs a critical biological function during development since disruption of the gene is embryonically lethal [40,41]. Transient transfection of an expression construct for Nrf1 into COS-1 cells can both *trans*-activate transcription from the promoter of the gene for GCS<sub>h</sub> and cause a concomitant increase in intracellular glutathione [42]. Evidence that Nrf1 contributes *in vivo* to protection against oxidants has been obtained from the finding that fibroblasts derived from embryos of mice bearing a targeted disruption of *Nrf1* have a diminished expression of both GCS<sub>l</sub> and glutathione synthetase, and this is accompanied by an increased sensitivity to paraquat and CdCl<sub>2</sub> [43].

The contribution of Nrf2 to the regulation *in vivo* of detoxication and antioxidant genes has been evaluated not only by examining the phenotype of cells that overexpress this bZIP protein, but also by examining cells and tissues that lack the factor. Transient transfection of wild-type Nrf2 into HepG2 cells results in increased reporter activity from GCS<sub>h</sub> and GCS<sub>l</sub> promoter transgenes [44]. Stable transfection of L929 cells with a regulatable dominant negative mutant form of Nrf2 was shown to diminish substantially the induction of HO-1 by CdCl<sub>2</sub>, ZnSO<sub>4</sub>, sodium arsenite and t-BHQ [45]. Fibroblasts from *Nrf2* (–/–) mice were demonstrated to express only about 15% of mRNA for GCS<sub>h</sub> and GCS<sub>l</sub> when compared with fibroblasts from *Nrf2* (+/+) mice [46]. The decreased expression of GCS subunits in *Nrf2* (–/–) fibroblasts was also reflected by the level of GSH which was only about 50% of that in fibroblasts expressing the factor [46]. Macrophages from *Nrf2* (–/–) mice have been shown to exhibit impaired expression of HO-1, peroxiredoxin MSP23, the 60 kDa stress protein A170, and the cysteine membrane transporter (system X<sub>c</sub><sup>–</sup>) [47]. The liver, forestomach and small intestine from *Nrf2* (–/–) mice have been found to exhibit reduced constitutive and/or inducible expression of Nqo1, aflatoxin B<sub>1</sub> aldehyde reductase, Gsta1/2, Gsta3, Gsta4, Gstm1, Gstm5, Gstp1/2, Ugt1a6, microsomal epoxide hydrolase, GCS<sub>h</sub>, manganese superoxide dismutase and catalase [12,48–50].

A major *in vivo* consequence of failure to express Nrf2 is increased sensitivity to xenobiotics. In many instances, this phenotype is probably due to an impaired ability to resynthesize GSH following depletion by reactive chemicals. The *Nrf2* (–/–) mice more readily develop liver damage caused by acetaminophen than wild-type mice [51,52]. Also, the *Nrf2* (–/–) mice readily develop pulmonary injury following treatment with butylated hydroxytoluene [53]. Like the liver and gastrointestinal tract, the lungs of the knockout mice have been found to contain diminished levels of mRNA for Nqo1, Ugt1a6, GCS<sub>l</sub>, HO-1, superoxide dismutase and catalase. In the context of cancer chemoprevention, the most important finding in *Nrf2* (–/–) mice is that the dithiolethione chemopreventive agent, oltipraz, fails to protect against benzo(a)pyrene-initiated cancer of the forestomach [54]. Furthermore, the mutant mouse develops a larger number of tumours than do wild-type mice exposed to benzo(a)pyrene that have not received treatment with oltipraz [54]. This suggests that Nrf2 controls constitutive protective mechanisms against xenobiotics.

To our knowledge, nothing has been published about the contribution of other CNC bZIP transcription factors to the regulation of the ARE gene battery. However, red blood cells from p45 NF-E2 deficient mice are sensitive to oxidative stress and show loss of expression of Nqo1, Gsta3 and catalase [55]. Further work is required to determine whether Nrf3, Bach1 or Bach2 contribute to the regulation of detoxication and antioxidant genes.

## 5. Transcription factors recruited to the ARE

Both Nrf1 and Nrf2 bind target DNA sequences as heterodimers with other bZIP proteins. Initial characterization of p45 NF-E2 demonstrated that it bound DNA as a heterodimer with p18 NF-E2, also called MafK [32,56]. Using p45 NF-E2 as a paradigm, it seems likely that Nrf1 and Nrf2 heterodimerize with members of the small Maf protein family, MafF, MafG and MafK. Evidence that Nrf1 and Nrf2 can associate with small Maf proteins has been provided by electrophoretic mobility shift assays and immunoprecipitation experiments [20,48,57–59].

In addition to being able to form heterodimers with

small Maf proteins, Nrf1 has been reported to dimerize with c-Jun, activating transcription factor 2 (ATF2) and ATF4 [60,61], and Nrf2 has been reported to dimerize with c-Jun, ATF4, PMF and PPAR $\gamma$  [62–65]. The physiological significance of Nrf1 and Nrf2 dimerizing with different bZIP proteins has received little attention to date. Transfection of MafG and MafK into cell lines negatively regulates ARE-driven gene expression [20,58], and it is therefore reasonable to suppose that Nrf1- and Nrf2-containing complexes will differ in the magnitude of transactivation depending on the relative amount of small Maf proteins present. Significantly, the bZIP factors MafG, ATF3, ATF4, c-Jun, JunB, Fra1 and Fra2 are themselves inducible by H<sub>2</sub>O<sub>2</sub> [66], CdCl<sub>2</sub> [63] and t-BHQ [67], and this will presumably introduce a temporal dimension into transcriptional activation through the ARE. It is anticipated that following drug exposure, the complexes recruited to the ARE, and related enhancers, will change with time.

Sequences that flank the ARE consensus can influence its function [16,17]. For example, a number of gene promoters contain an ARE-related motif located close to the core enhancer that regulates basal expression. In the case of *rGSTA2* and *mGsta1*, these two sequences are tandemly arrayed, whereas in *rNQO1* and *hNQO1*, they are in reverse orientation and can form a 13 bp palindrome [1,3,23]. It is possible that the sequence context of different AREs will influence the bZIP dimers that are recruited to the promoter. Certainly, Nguyen et al. [20] have shown that rNrf2 binds the ARE in the *rNQO1* gene promoter more avidly than the ARE in the *rGSTA2* promoter. The binding specificity of different bZIP dimers towards palindrome-type and tandemly arrayed AREs ought to be investigated.

## 6. Intracellular sensors of chemopreventive blocking agents and transduction of a chemical stress signal to the ARE

The actin-binding protein Keap1 has been identified as a docking site for Nrf2 that is responsible for sequestering the bZIP protein in the cytoplasmic compartment of unstressed cells [68]. The association between the two proteins is between the C-terminal DGR domain of Keap1 and the N-terminal Neh2 domain of Nrf2. It is

worth noting that Nrf1 also contains a Neh2 domain, and therefore, it too probably interacts with Keap1 in vivo. A model depicting the movement of Nrf2 between compartments in non-stressed and chemically-stressed cells is shown in Fig. 3.

The mechanisms by which cells recognize the presence of chemopreventive blocking agents remain to be elucidated. However, a key feature of these chemicals is that they interact with thiol groups, and would therefore be expected to modify cysteine residues within proteins [1,10]. It is therefore possible that the modification of cysteine residues within Keap1 or Nrf2 could serve to trigger the release of the bZIP protein from its tethering site. An alternative possibility discussed previously [8,69] is that the active site cysteine residue of protein kinase phosphatases could be modified by blocking agents, and their resulting inactivation could allow protein kinase signalling to proceed unchecked. An example of this is provided by receptor-directed tyrosine phosphatases being inactivated by UV irradiation, oxidants and alkylating agents [70,71]. Similarly, certain class Mu and class Pi GST subunits have been reported to act as endogenous inhibitors of stress-activated protein kinases [72,73]. It can therefore be postulated that in the presence of chemopreventive agents, GSTM1 and GSTP1 relax their inhibition of protein kinase activity, possibly through either covalent or non-covalent binding of the xenobiotic by the transferase.

Three of the mitogen activated protein (MAP) kinases, extracellular-regulated kinase (ERK), p38, and c-Jun N-terminal kinase (JNK), have been implicated in ARE activation [74–76]. Two lines of evidence link MAP kinases to the ARE response. Firstly, many of the xenobiotics that stimulate ARE-driven gene expression also activate protein kinase activity. Secondly, modulation of MAP kinase activity by pharmacological inhibition, knockout by dominant negative mutants, or overexpression of constitutively active mutants modifies ARE-driven reporter activity. The emerging picture is complex, and suggests that the involvement of specific MAP kinases is variable and may be ARE-specific, inducer-specific and tissue-specific. Such variation is well illustrated by reference to the p38 kinase. Kong and his colleagues demonstrated that in serum-starved human HepG2 cells and murine Hepa1c1c7 cells, both

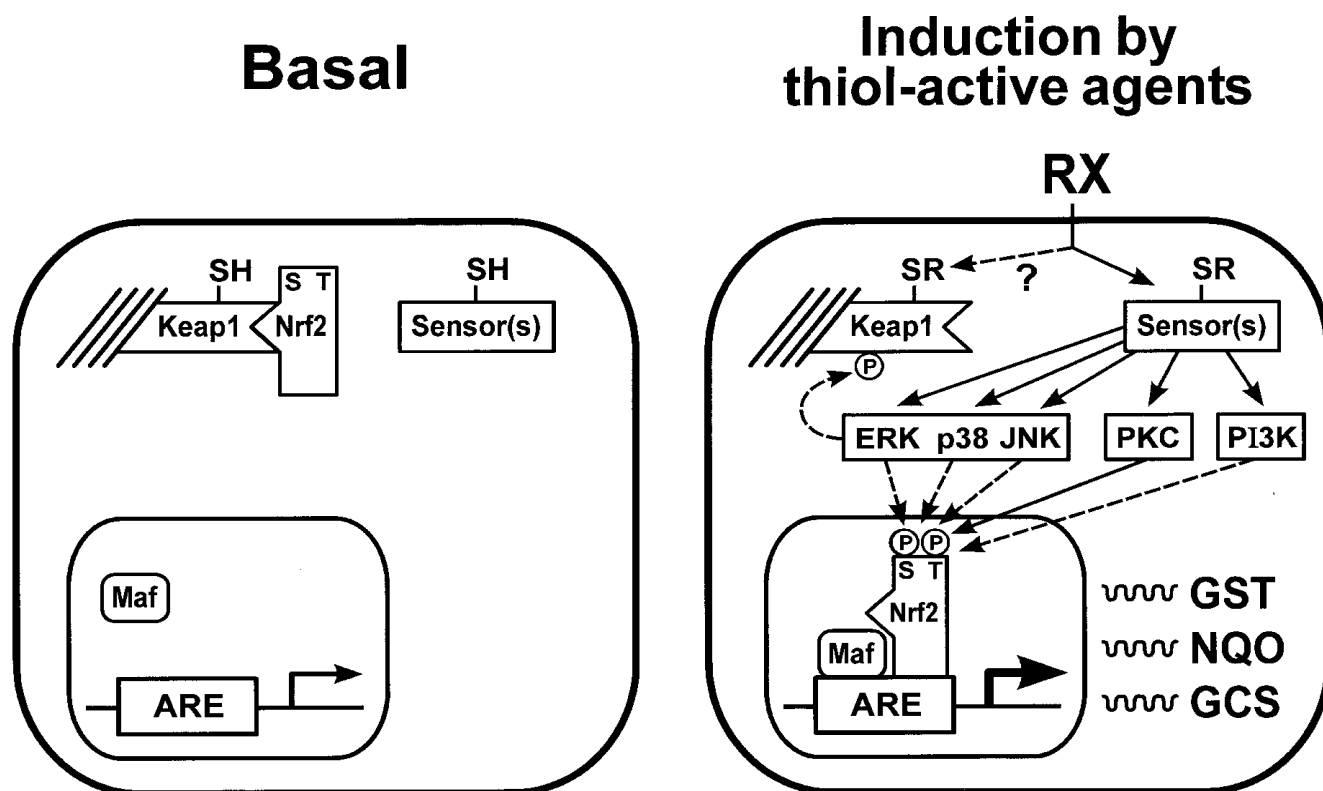


Fig. 3. Model of signalling pathways involved in activation of Nrf2 by chemopreventive blocking agents. The left-hand panel shows that under basal conditions, Nrf2 resides in the cytoplasm tethered to Keap1, whereas small Maf proteins are constitutively nuclear. The right-hand panel shows that upon treatment with blocking agents, the activities of MAP kinases, PKC and PI3K are increased, presumably in response to thiol-dependent stimulation of 'sensors' that recognize the presence of specific classes of xenobiotic [69–82]. Activation of protein kinases leads to release of Nrf2 from Keap1, and translocation of the transcription factor to the nucleus where it may dimerize with a small Maf protein or another bZIP protein, before binding ARE enhancers in the promoters of inducible genes [68]. The signalling pathways responsible for release of Nrf2 from Keap1 have not been defined (shown by hatched lines) and may involve phosphorylation of either protein. Equally, it could entail phosphorylation of the dimerizing partner for Nrf2. Evidence has been presented that PKC can phosphorylate Nrf2 [83], and this is therefore shown by a solid line.

t-BHQ and  $\beta$ -NF, but not the isothiocyanate sulforaphane, induced p38 activity [75]. These workers found that the induction by t-BHQ of transcription from the *mGsta1* ARE was augmented in these liver cell lines by pre-treatment with the p38 inhibitor SB203580, suggesting that p38 negatively regulates the ARE [75]. By contrast, activation of the human HO-1 gene ARE by  $\text{CdCl}_2$  in human mammary MCF-7 cells is diminished by pre-treatment with SB203580 [27]. Finally, in the rat hepatoma H4IIE cell line, both t-BHQ and sulphur amino acid deprivation (SAAD) activated p38 [77,78]. Nonetheless, the resulting induction of rGSTA2 mRNA by SAAD, but not by t-BHQ, was attenuated by SB203580.

At present, it is unclear how MAP kinases impinge upon ARE activation, and whether PI3K is essential for the response [74–79]. Of the proteins with clearly defined roles in ARE activation, none can be described definitively as MAP kinase substrates. However, Nrf1, Nrf2 and Keap1 contain potential proline-directed serine/threonine residues, and therefore cannot be disregarded as possible substrates. Similarly, certain small Maf proteins also contain proline-directed serine and threonine residues, and therefore, might be phosphorylated by MAP kinases. Both c-Jun and ATF2 are substrates for JNK [80–82]. Thus, it is reasonable to ascribe some of the variable results noted above to tissue-specific differences in

signalling pathways, and differences in the composition of bZIP heterodimers recruited to the ARE enhancers in the promoters of different genes.

Whilst MAP kinase signalling to the ARE may be mediated by the dimerization partners of Nrf1 or Nrf2, it should be recognized that these latter CNC bZIP factors are phosphorylated *in vivo*. It has recently been demonstrated that treatment of HepG2 cells with t-BHQ results in increased phosphorylation of Nrf2, and that this is associated with appearance of the transcription factor in the nucleus [83]. Evidence suggests that the phosphorylation in HepG2 cells is largely mediated by protein kinase C (PKC). *In vitro*, Nrf2 is an excellent substrate for PKC [83]. Furthermore, PKC activity can be augmented in HepG2 cells by t-BHQ treatment. Crucially, pre-treatment of HepG2 cells with staurosporine, a pharmacological inhibitor of PKC, has been reported to abolish both phosphorylation and nuclear translocation of Nrf2 in response to t-BHQ [83].

## 7. Concluding comments

In this review, we have highlighted recent advances in our understanding of how cancer chemopreventive blocking agents transcriptionally activate gene expression. This response to blocking agents appears to represent cellular adaptation to chemical stress. Although it is clear that Nrf1 and Nrf2 play pivotal roles in transcriptional activation of cytoprotective genes, much remains to be learnt about how their dimerizing partners might influence this process. It seems likely that a large number of bZIP heterodimers can bind the ARE core enhancer, and therefore there is potential for both subtlety and specificity in the response to chemopreventive agents that is at present poorly understood. Relatively little is known about the number of genes that can be included in the ARE gene battery. This is an area that warrants investigation since the existence of inducible transcription factors that appear to be regulated through ARE-like elements may result in distinct batteries of genes being indirectly inducible by chemopreventive blocking agents.

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